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CONCERNING SO-CALLED AGGLUTINOIDS.*

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THE agglutination reaction was first described as a specific reaction by Gruber and Durham in 1896. It has since been the subject of much investigation, which need not be recapitulated here. German investigators soon noticed that in making tests with serial dilutions agglutination might fail to appear at certain dilutions, although distinct at higher dilutions. These portions of a serial dilution where the agglutination failed to appear have been called inhibition areas (*Hemmungszonen*), and following Ehrlich a supposititious substance has been regarded as the cause of this failure, much as the ancients were inclined to assume special causes for all the different phenomena of nature. The body which prevents the expected action of the agglutinin is called "agglutinoid."

I have studied some antityphoid sera, which showed these phenomena nicely (Table 1). In this table, *s* = a slight reaction; + = a distinct reaction; *c* = a complete reaction, that is, the liquid is cleared and all the germs settled to the bottom.

TABLE 1.

ANIMAL	DATE		DILUTION OF SERUM														
	Bleeding	Test	1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1 280	1:5,000	1:10 000	1:20 000	1:40,000	1:80,000	1:160,000
	Dec.	Dec.															
Sheep No. 56	11	11	o	s	c	c	c	c	+	o	o	o	o	o	o	o	o
Sheep "Stub"	10	10	o	o	o	+	+	c	c	c	c	c	c	c	o	o	o
Black goat	10	10	o	o	o	o	o	o	o	+	c	c	c	+	o	o	o
Bay horse	4	10	+	+	+	s	s	c	c	c	c	c	c	c	c	+	o
Sorrel horse	10	10	c	c	c	o	o	o	o	+	c	c	c	c	+	+	o

The serum from Sheep 56 did not agglutinate in a dilution higher than 1:320 and in low dilutions, as 1:10, agglutination was incomplete and in 1:5 entirely absent. In the serum from Sheep "Stub," agglutination was obtained in dilutions as high at 1:20,000, and the inhibitory area also extended farther up, so that complete agglutination is first seen in the dilution 1:160. In the serum from the black goat, although the final dilution is only 1:20,000, agglutination is first seen at 1:640. It will readily be seen that should a typhoid patient possess a serum like this, the ordinary clinical methods of performing the agglutination test would not detect it. In the serum from the bay horse, the final dilution extends to 1:80,000 and now some agglutination occurs in the lower portion of the inhibition area. In the serum from the sorrel horse, we have the same phenomenon except that here the agglutination is complete and perfect in the low dilutions, absent for a space, then again complete.

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Some experiments have been made to throw light if possible on the cause of these phenomena. Table 2 shows the tests made to determine the influence, if any, that the presence or absence of complement might exert. The results show that neither the entire absence of complement (heated at 56° C. for one-half hour) nor an excess of complement (either from horse or guinea-pig) made any appreciable difference in the results. In the test with guinea-pig serum *l* indicates complete lysis of the germs.

TABLE 2.

SERUM	DILUTION OF SERUM														
	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:5,000	1:10,000	1:20,000	1:40,000	1:80,000	1:160,000	1:320,000
Antityphoid	c	c	o	o	o	o	c	c	c	c	c	c	+	o	o
Inactivated 56° C. ½ Hr.	c	c	o	o	o	o	c	c	c	c	c	c	+	o	o
Antityphoid $\frac{1}{5}$ Normal horse $\frac{4}{5}$	c	c	+	o	o	o	c	c	c	c	c	c	+	o	o
Antityphoid $\frac{1}{5}$ Guniea-pig $\frac{4}{5}$	l	l	o	o	o	o	c	c	c	c	c	c	+	o	o
Water $\frac{1}{5}$ Normal horse $\frac{4}{5}$	c	c	+	c	c	c	o	o	o	o	o	o	o	o	o

TABLE 3.

ANIMAL	DATE		DILUTION OF SERUM														
	Bleeding	Test	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1 280	1:5,000	1:10 000	1:20 000	1:40 000	1:80 000	1:160 000	1:320,000
	Dec.	Dec.															
Sorrel horse	17	18	c	o	o	o	s	s	c	c	c	c	c	c	o	o	o
Goat No. 76	15	18	l	o	+	+	+	+	c	c	c	c	+	s	o	o	o
Goat No. 76	16	18	l	o	o	+	+	+	c	c	c	c	+	+	o	o	o
Goat No. 76	17	18	l	o	o	+	+	+	c	c	c	c	+	s	o	o	o

The animals yielding these sera were being injected twice a week, and I tested the serum from one animal daily to determine what effect, if any, the injection might have. Table 3 shows no appreciable change.

I now turned to a study of the effect, if any, that the age of the culture might have. Table 4 shows that the age of the culture affects the agglutinating power, a 24-hour culture on agar being agglutinated in twice as high a dilution as a 15-hour culture, while a five-day broth culture is similarly more agglutinative than a 24- or 15-hour culture. The inhibition area is, however, more marked in the younger cultures.

TABLE 4.
AGE OF CULTURE.

CULTURE		DILUTION OF SERUM													
		1:12½	1:25	1:50	1:100	1:200	1:400	1:800	1:1,600	1:3,200	1:6,400	1:12,800	1:25,600	1:51,200	1:102,400
Grown on agar	15 hrs...	3	4	5	5	3	2	1	0	0	0	0	0	0	0
	24 hrs...	5	5	5	6	6	5	4	2	2	1	1	0	0	0
Grown in broth	15 hrs...	3	3	4	5	4	3	1	1	0	0	0	0	0	0
	24 hrs...	4	5	5	5	4	3	1	0	0	0	0	0	0	0
	5 days..	5	5	5	3	5	5	4	4	4	4	2	1	0	0

In these tests the degree of agglutination is indicated by the numerical system: the higher the number, the more complete the agglutination. In interpreting an agglutination reaction, there are two factors to be considered: (1) the granules, floccules, or sediment; and (2) the cloudiness or clearness of the fluid. Of course, these phenomena are inversely proportional to each other and I have found that recording each separately enables one to indicate differences that otherwise would escape being recorded. We may distinguish five degrees of each and indicate them by numbers from 0 to 4, as shown:

Flocculation	Number	Cloudiness	Old System
None	0	complete	no reaction
Slight	1	abundant	slight
Distinct	2	distinct	+
Abundant	3	slight	+
Complete	4	none	complete

In recording a reaction both the flocculation and the residual cloudiness are recorded independently and then added.

The results with the age of the cultures, shown in Table 4, seem to indicate that some substance produced during the growth of the germs and which tends to accumulate is connected with the agglutination reaction. The slower diffusion in agar might account for the more rapid accumulation of this substance in agar cultures.

Some have contended that agglutination is really produced by a precipitation, the germs being caught and carried down in the precipitate. Now, if this hypothetical substance, which thus favors agglutination, is outside the germ body, we should be able to wash it away. To test this, I have removed the growth from an agar culture with physiological-salt solution, emulsified thoroughly with a mechanical shaker, and recovered the bacilli by centrifugalization; these were again emulsified in salt solution and allowed to stand 20 hours. At the end of 20 hours, the bacilli were recovered by the centrifuge, again emulsified, again recovered, and then made up into a suspension of the usual density and used at once. The physiological-salt solution used in all this work contained 0.4 per cent of formaldehyde to prevent germ growth. Table 5 shows that agglutination occurred as usual and that the hypothetical substance could not be removed by such means.

If the substance were in the germ body, it might possibly be removed by the plasmolytic action of distilled water; hence, I repeated the washing as before, except that distilled water was used in place of physiological-salt solution. Table 5 shows that practically no agglutination occurred. However, this did not show that I had removed from the germ bodies a hypothetical something necessary to agglutination, for upon adding salt to these tubes they agglutinated as usual. I had merely shown that in some way salt is necessary to agglutination.

TABLE 5.

EFFECT OF WASHING.

	DILUTION OF SERUM											
	1:12½	1:25	1:50	1:100	1:200	1:400	1:800	1:1 600	1:32,000	1:64,000	1:12,800	1:25,000
NaCl sol	4	5	5	6	6	5	4	3	3	1	1	0
Distilled water	½	½	½	½	0	0	0	0	0	0	0	0

Many other tests of various kinds were made without any illuminating results. However, in working with different suspensions from day to day, it was found that the density of the suspension influences the character of the reaction. Table 6 shows this distinctly. It will be observed that a total inhibition area occurs only in the dilute suspension. Further, that, while the final limit of agglutination is not greatly changed, the limit of complete reaction is distinctly changed, being lower in the denser suspensions. The point of beginning complete reaction is also lowered, so that the effect is to lower or push

TABLE 6.

DILUTION OF EMULSION	DATE		DILUTION OF SERUM													
	Bleeding	Test	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:5,000	1:10,000	1:20,000	1:40,000	1:80,000	1:160,000
1:4	Dec. 17	Dec. 22	+	o	o	o	s	s	c	c	c	c	c	c	+	o
1:2	17	22	+	+	+	+	+	c	c	c	c	+	+	+	+	o
1:1	17	22	+	+	+	c	c	c	+	+	+	+	+	+	o	o

TABLE 7.

DILUTION OF SUSPENSION	DILUTION OF SERUM											
	1:12½	1:25	1:50	1:100	1:200	1:400	1:800	1:1,600	1:3,200	1:6,400	1:12,800	1:25,600
1:32	8	o	o	o	o	4	5	8	8	8	6	o
1:16	8	2	2	4	4	8	8	8	8	6	2	o
1:8	6	6	6	6	8	7	4	4	4	4	1	o
1:4	6	6	6	8	7	6	6	4	4	4	2	o
1:2	6	6	7	8	5	4	3	3	3	1	1	o
1:1	7	8	8	8	6	3	2	2	2	1	o	o

TABLE 8.

NUMBER	$HgCl_2$	KI	WATER	RESULTS	FINAL DILUTION	
					$HgCl_2$	KI
1	1 c.c. n/10	1.0 c.c. n/1	0.0 c.c.	–	n/20	n/2
2	"	0.4 "	0.6 "	–	"	n/5
3	"	0.16 "	0.84 "	+	"	n/12½
4	"	1.0 c.c. n/10	0.0 "	+	"	n/20
5	"	0.4 "	0.6 "	+	"	n/50
6	"	0.16 "	0.84 "	s	"	n/125
7	"	1.0 c.c. n/100	0.0 "	–	"	n/200
8	"	0.4 "	0.6 "	–	"	n/500
9	"	0.16 "	0.84 "	–	"	n/1,250

TABLE 9.

DILUTIONS OF $HgCl_2$	DILUTIONS OF KI											
	5n	2n	$\frac{4}{5}$n	n/2	n/5	n/12$\frac{1}{2}$	n/20	n/50	n/125	n/200	n/500	n/1,200
n/200	–	–	–	–	–	–	–	+	+	+	–	–
n/20	–	–	–	–	+	+	+	+	+	–	–	–
n/2	–	–	+	+	+	+	+	–	–	–	–	–

TABLE 10.

	DILUTION OF SERUM								
	1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280
Suspension of typhoid bacilli	o	+	c	c	c	c	+	o	o
	DILUTION OF HCl								
	n/1	n/2	n/4	n/8	n/16	n/32	n/64	n/128	n/250
Suspension of staphylococci	o	+	c	c	c	c	c	+	o

TABLE 11.
(BUXTON AND RAHÈ.)

DILUTIONS OF MASTIC	DILUTIONS OF $Fe(OH)_3$								
	1:10	1:500	1:1,000	1:2,000	1:3,000	1:5,000	1:8,000	1:16,000	1:20,000
1:300	–	–	–	–	–	+	++	Tr	–
1:200	–	–	–	–	–	+++	+++	Tr	–
1:120	–	–	–	–	+++	+++	+++	–	–
1:60	–	–	–	+++	+++	+++	–	–	–
1:10	–	–	+++	+++	+++	–	–	–	–

back the area of complete reaction in the denser suspensions. Table 7 shows a similar result when using a greater range of densities and the numerical notation. Now, this phenomenon is important in that it is comparable to well-known chemical phenomena: when bichloride of mercury and potassium iodide are mixed in certain proportions, the result shown in Table 8 is obtained. Here the bichloride, corresponding to the emulsion, maintains a constant dilution (n/20), while the KI, corresponding to the serial dilution of serum, varies from n/2 to n/1,250. A precipitate occurs or the reaction is positive only within certain limits (n/12½ to n/125), as is the case in agglutination. In the chemical reaction we say the precipitate is soluble in excess of either reagent, whatever this may mean. In agglutination we do not know what to say. Now, when the concentration of the bichloride is varied, we get the same lowering or pushing back of the area of positive reaction as in agglutination (see Table 9).

Again, various chemicals will agglutinate germs. Note in Table 10 the resemblance between the agglutination with hydrochloric acid and the agglutination with a specific antiserum. In agglutination with the acid the germs must be suspended in a salt-free medium in contrast to the serum which requires the presence of salt. In agglutination with the acid one sees an inhibition area as well marked as with the serum.

It must be admitted that from hydrochloric acid and the bichloride of mercury to serum is a long cry and the value of these resemblances might be questioned; but in the flocculation of colloids we encounter a phenomenon much more nearly resembling the agglutination of germs and which may serve as a connecting link between them.[1]

In Table 11 it will be seen that when colloidal mastic is flocculated with colloidal $FeCl_3$, varying the dilution of the mastic causes the zone of reaction to be pushed back exactly as is the case with a suspension of germs or with bichloride of mercury.

Again, Table 12, also taken from Buxton and Rahè's article, shows that the flocculation of colloidal platinum with $FeCl_3$, of colloidal mastic with $FeCl_3$, of colon germs with $FeCl_3$, and of typhoid germs with a specific antiserum is of the same type throughout, and shows a well-marked inhibition area.

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[1] Buxton and Rahè, *Jour. Med. Research*, 1909, 20, p. 113.

It is thus shown that the peculiar inhibition areas found in agglutination with a specific antiserum are not unique, but that similar phenomena can be traced down through organic colloids and inorganic colloids to simple inorganic chemistry. Hence, although we do not yet understand the cause of the phenomena, we are no longer justified in assuming the existence of a special causative agent—the so-called "agglutinoids."

TABLE 12.
(Buxton and Rahè.)

Colloidal platinum and $FeCl_3$	Dilutions	n/1	n/10	n/100	n/500	n/5,000	n/6,000	n/10,000	n/12,000	n/20,000		
	Results	+	+	+	–	–	+	+	+	–		
Mastic and $FeCl_3$	Dilutions	n/1	n/20	n/40	n/500	n/1,000	n/2,000	n/3,000	n/4,000	n/6,000	n/8,000	
	Results	+	+	–	–	–	+	+	+	+	–	
B. coli communis $FeCl_3$	Dilutions	n/1	n/20	n/200	n/300	n/400	n/600	n/1,000	n/1,500	n/2,000	n/3,000	n/4,000
	Results	–	+	+	+	–	–	–	+	+	+	+
B. typhosus and serum	Dilutions	1:5	1:10	1:20	1:40	1:320	1:640	1:1,280	1:5,000	1:20,000	1:80,000	1:160,000
	Results	+	+	+	–	–	+	+	+	+	+	–